

## SURVIVAL STRATEGIES OF MICROORGANISMS IN EXTREME SALINE ENVIRONMENTS

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### ABSTRACT

Halophilic representatives are found in all main lines of evolutionary descent of microbes: in archaeobacteria, Gram-negative and Gram-positive eubacteria, and also in eucaryotes. In principle all halophilic microorganisms have to adapt their surface and membrane structures to their highly ionic environments. Concerning their intracellular compartment two different strategies have been developed: Inorganic ions are largely excluded in some microorganisms while such ions are actively accumulated in others. In particular the second group of organisms has to adapt the whole metabolic machinery to the highly ionic conditions of several molar salts, whereas in the first group only the outer surface of the cytoplasmic membrane and the extracytoplasmic structures are in contact with high concentrations of inorganic ions. In this latter group, a variety of organic solutes is accumulated in response to increases of the salinity of the environment.

### INTRODUCTION

Most of the aquatic microorganisms that live on earth inhabit freshwater and marine habitats and quite a small number tolerate or even require moderate to high salt concentrations. Those microorganisms that have developed mechanisms to survive and to live in hypersaline and extremely concentrated brines enjoy decreasing competition with other microorganisms because of a decreasing species diversity with increasing salinity. In addition many predators (with the important exception of the brine shrimp *Artemia salina*) can not survive under these conditions and therefore halophilic bacteria often accumulate in masses in hypersaline brines.

### ENVIRONMENTAL FACTORS

In nature, hypersaline environments are present on all continents, although they are predominant in warm and dry climatic regions. The major components of the inorganic ions contributing to the total salinity of some representative salt lakes are summarised in Table 1. From these data it is apparent, that not only the relative proportions of bivalent cations (particularly magnesium and calcium), but also those of the anions (particularly chloride, sulfate and carbonate) vary to a high degree.

Adaptation and survival of microorganisms in these habitats is not only dependent on the total salt concentration, but also on the relative ionic composition of the environment. In this respect, it is interesting to see that among the halobacteria, species have developed that are specifically adapted to the thalassohaline environment (see /1-3/) or to the Dead Sea with its extraordinary high content of calcium and magnesium ions /4,5/. Both environments have a near neutral pH. The existence of forms specifically adapted to magnesium and calcium deficient, alkaline environments has been postulated after observations in some soda lakes in the Vadi Natrun, Egypt /6/. Such bacteria have been isolated from these and similar lakes /7-9/.

Similar adapted forms may be found in the algal genus *Dunaliella*, which lives in the Dead Sea /10/, in the Great Salt Lake /11/, in alkaline soda lakes /6/, and also in marine salterns /12/.

Halophilic *Halobacterium* cells, however, appear specifically adapted to alkaline brines and mass developments of these bacteria have not been found in salt lakes with neutral pH. Despite this variation in the relative ionic composition of natural salt lakes, the general problem of all halophilic microorganisms is to successfully deal with high ionic concentrations and high osmotic pressures created by these salt solutions.

## SALT RELATIONS OF HALOPHILIC MICROORGANISMS

The organisms which occur in extreme saline environments include Gram-negative and Gram-positive eubacteria, archaeobacteria and even eucaryotic microorganisms like the alga Dunaliella, all of which are on different main lines of microbial evolution that show significant principal physiological and structural differences, such as in the composition of their cell membranes and outer cell layers. Consequently structural adaptation in these different groups is expected to reveal differences, even if similar principal strategies are employed.

The microorganisms most commonly found in many brines include first of all the halobacteria (archaeobacteria which perform aerobic respiratory metabolism), the Ectothiorhodospiraceae (Gram-negative phototrophic bacteria living in the anoxic environment of alkaline salt lakes), the alga Dunaliella (an oxygen producing, phototrophic eucaryote without a cell wall), and occasionally the cyanobacterium Spirulina (which forms blooms in some soda lakes, but is less halophilic than the before mentioned microorganisms). All of these microorganisms have the capacity to transform light energy into metabolically usefull energy, i.e. membrane gradients and ATP. The halobacteria use a light driven proton pump in their purple membrane, while all others have chlorophyll mediated reactions. Probably the coloration of these microorganisms makes their development so conspicuous and therefore, more easily recognized than that of non-colored heterotrophic bacteria which have been found in high numbers in marine salterns of intermediately high salt content, where Dunaliella also reaches the highest cell density /12/.

TABLE 1: Ionic Composition of Different Types of Salt Lakes

	Great Salt Lake	Marine saltern	Soda lakes Zugm	Gaar	Dead Sea
Na <sup>+</sup>	105.4	65.4	142.0	137.0	39.2
K <sup>+</sup>	6.7	5.2	2.3	1.4	7.3
Mg <sup>2+</sup>	11.1	20.1	nd.	nd.	40.7
Ca <sup>2+</sup>	0.3	0.2	nd.	nd.	16.9
Cl <sup>-</sup>	181.0	144.0	154.6	173.7	212.4
SO <sub>4</sub> <sup>2-</sup>	27.0	19.0	22.6	48.0	0.5
Carbonates	0.7	-	67.2	6.6	0.2
Total salts	332.5	253.9	393.9	374.2	322.6
pH	7.7	ca. 7	11.0	10.9	ca. 6

Concentrations are in g/l; nd. = not detected; data from /6,13-15/.

According to the amount of salt required for optimal growth and to the upper limit of salt concentrations that still allows growth, we distinguish between halophily and halotolerance. Bacteria are classified as non-halophilic with salt optima below 0.2 M NaCl, as slightly halophilic with optima between 0.2 and 1.0-1.2 M NaCl, as moderately halophilic with optima between 1.0 to 2.0-2.5 M NaCl and as extremely halophilic with optima above 2.5 M NaCl (which is approximately 15%, see /16/). The same limits of salt concentrations are used to describe their salt tolerance.

It is obvious from the comparison of their salt optima, that a number of halophilic microorganisms have developed mechanisms to optimally adapt to varying high salt concentrations and are stressed by lowering this salt content. On the one hand, 0.5 M NaCl is an extreme salt stress hardly tolerated by the non-halophilic E. coli. On the other hand, Halobacterium cutirubrum and Ectothiorhodospira halophila can barely survive, even if the salt concentration is doubled to about 1 M NaCl. Therefore, from the point of view of a Halobacterium, extremely high salt concentrations are quite normal and can not be regarded as a stress. This implies that all cellular structures and the whole metabolic machinery are well adapted to the highly ionic conditions and high osmotic pressures of their environments. In the following, different strategies of various microorganisms, in particular of Dunaliella, of halobacteria, and of various halophilic and halotolerant eubacteria, are briefly summarized.

### SURVIVAL MECHANISMS

The first problem that all halophilic microorganisms have to deal with is the osmotic balance, generally achieved by the accumulation of high internal solute concentrations. Increasing solute concentrations are equivalent to increasing water deficiency. Halophilic bacteria appear optimally adapted to this kind of water stress. Even *E. coli*, growing in very dilute media maintains a cytoplasmic osmotic pressure that approximately equals a 300 mM salt solution /17/, whereas halophilic microorganisms may accumulate several molar concentrations of a single solute. As pointed out by Brown /18,19/, it is a precondition for the accumulation of high solute concentrations within the cell that these solutes have to be compatible with cellular structures and physiological processes, i.e. they must not destroy protein and membrane structures and must be less inhibitory to enzymatic reactions than other solutes. Brown called these solutes "compatible solutes". A number of different compatible solutes is accumulated by various microorganisms (see below).

The second problem that faces extremely halophilic microorganisms is the structural and functional integrity of the cell membranes and outer layers that, in contrast to the cytoplasmic compartment, are in direct contact with the highly ionic environment. It is this part of the cell that mediates exchange of substrates and metabolic products between cytoplasm and external environment, catalyzes the built up and maintenance of high solute gradients, serves the energy generation of the cell, and gives the cell its structural rigidity.

#### The Alga *Dunaliella*

*Dunaliella* is the dominant primary producer of the northern part of Great Salt Lake (where the salt concentration reaches saturation) /11/, of the Dead Sea /10/, of Lake Gaar in the Wadi Natrun /6/, and also occurs in great numbers in ponds of marine salterns with immediately high salt concentrations /12/. *Dunaliella* quite effectively excludes inorganic ions from the cells and accumulates for osmotic compensation high concentrations of glycerol. In *D. viridis* growing at 4.25 M NaCl, glycerol is accumulated to a concentration of 4.4 molal /20/. Accumulation of glycerol is possible by photosynthesis as well as from accumulated starch in the dark. *Dunaliella* is able to quite effectively regulate its glycerol content in response to the external salt concentration. A glycerol cycle has been proposed to occur in this alga /21/ that involves formation of glycerol from dihydroxyacetone phosphate via glycerol-3-phosphate and degradation of glycerol to dihydroxyacetone and dihydroxyacetone phosphate, which then can be osmotically inactivated by conversion into storage carbohydrate. Evidence accumulates that both processes are spatially separated. The key step of the biosynthetic pathway is mediated by glycerol-3-phosphate dehydrogenase localized in the chloroplast, whereas the key step of its degradation is catalyzed by glycerol dehydrogenase localized in the cytoplasm /22,23/. This separation makes sense, as the required energy and photosynthetate is provided by the light metabolism in the chloroplast, whereas under conditions of decreased osmolarity, glycerol accumulated in the cytoplasm has to be removed rapidly from this compartment.

Little is known about the structural adaptation of this alga. In particular, the structural basis of the impermeability of the cytoplasmic membrane to glycerol, which is freely permeable through other biological membranes, needs to be studied.

#### The Halobacteria

Dominating types of heterotrophic bacteria in Great Salt Lake, Dead Sea, African soda lakes, and concentrated pools of marine salterns are various species of extremely halophilic bacteria (halobacteria) of the genera *Halobacterium*, *Halococcus*, *Natronobacterium*, *Natronococcus*, and *Haloarcula*. Halobacteria are without doubt the organisms which are best adapted to the life in the extremes of concentrated salt solutions. (The extremely halophilic species of *Ectothiorhodospira* are dependent on anoxic conditions.) In contrast to other halophilic microorganisms, their cytoplasm is highly ionic and KCl is the main compatible solute. Potassium concentration exceeding 5 M and sodium concentrations in the molar range have been reported /1,24-26/. The total internal concentration of monovalent cations even exceeds the external one.

**Proteins.** Many enzymatic reactions are dependent on high potassium concentrations and also tolerate high concentrations of NaCl /2,24,27/. One striking feature of halobacterial proteins is their highly acidic nature and it was argued, that high ion concentrations are required for neutralization of the protein charges to keep them in a metabolically active configuration. Charge shielding should, however, be effectively completed at monovalent cation concentrations well below 1 M, as has been pointed out by Lanyi /27/, whereas many halophilic enzymes require much higher concentrations for activity and stability. It was proposed that the high salt demand of these proteins is not only necessary for charge shielding, but that their stability depends on conditions where hydrophobic interactions are increased by high concentrations of NaCl or KCl /27/.

Cell envelope. Structural integrity of the cell envelope of Halobacterium cutirubrum is extremely sensitive to the ionic strength of the medium. When cells are suspended in successively lower NaCl concentrations, they change from rods to spheres and lyse at about 1.0 to 1.5 M NaCl (see /25/). Disintegration of the cell membrane occurred at somewhat lower NaCl concentrations below 1 M and could be prevented by 20 mM magnesium even in the complete absence of NaCl /28/. The major component of the cell wall of H. salinarium is a glycoprotein of high molecular weight and it was suggested that the carbohydrate moiety of this glycoprotein is essential for maintenance of the cell shape /29/.

Lipids. About 2-4% of the cell dry weight in H. cutirubrum are lipids. The polar lipids of these bacteria, consisting of phospholipids and glycolipids, compose about 90% of the total lipids. These polar lipids are derivatives of a glycerol diether, and usually have phytanyl groups as hydrocarbon chains /9,30,31/. In alkaliphilic halobacteria  $C_{20}C_{25}$  and  $C_{25}C_{25}$  diether core lipids have also been found /9,31/. The major lipids of H. cutirubrum are diphytanyl glycerol ether analogs of phosphatidylglycerophosphate, phosphatidylglycerol, and phosphatidylglycerosulfate. In addition to these phospholipids several halobacteria have glycolipids, in particular unusual sulfated glycolipids. The neutral lipids consist mostly of squalene, dihydro- and tetrahydrosqualene, MK-8, diphytanol glycerol ether, and various pigments /32-34/. At neutral pH, a lipid such as phosphatidylglycerophosphate is a dianion with each phosphate group singly ionized /35/. It was pointed out that in a bilayer structure, one of these phosphate groups will be at the aqueous surface, whereas the other will be in the bilayer structure and therefore less accessible to water and cations /36/. From their observations, these authors proposed that magnesium ions can form bridges to the outer charged headgroups of the polar lipids only if sufficient neutral lipid like squalene is present to space the phospholipids far enough apart to allow cations to approach the negatively charged groups. The active participation of protein would also be necessary for the stability of such bilayers. The stability of the membrane bilayer of the classical halobacteria at high salt concentrations seems therefore to depend on the combination of their polar lipids, neutral lipids, bivalent cations, and proteins. The participation of bivalent cations in membrane stabilization is, however, unlikely in the alkaliphilic halobacteria (species of Natronococcus and Natronobacter) which are adapted to environments deficient in those cations. These bacteria grow well at 0.1-2.0 mM magnesium and are inhibited at higher concentrations /7,8/. It is necessary to assume that either monovalent cations replace the function of bivalent cations or that negative charges are screened by organic molecules in these bacteria.

#### The Halophilic Eubacteria

Proteins. The high acidity of proteins in halobacteria has been explained as an adaptive mechanisms towards their high content of ions in the cytoplasm. Since inorganic ions are largely excluded from the cytoplasm of halophilic eubacteria (see below), one would predict that soluble proteins of these bacteria are much less acidic. Membrane proteins, however, which have one side exposed to the highly ionic environment, should have an acidity intermediate between those proteins of halobacteria and of non-halophilic bacteria. An analysis of membrane proteins of several Ecotiorhodospira strains with different optimal salinities revealed an increasing amount of polar and intermediate amino acid residues, concomitant with a decrease of apolar residues, in proportion to their increasing salt requirement /37/. The results with whole cell proteins did not show this clear correlation. As discussed by Lanyi /27/ the intermediate amino acid residues are thought responsible for the stability of halophilic enzymes at high salt concentrations.

It was shown recently, that whole cells of the anaerobic halophilic bacteria Haloanaerobium praevalens, Halobacteroides halobius, and Clostridium lortetii, contain proportions of polar amino acids more similar to Halobacterium than to E. coli cells and have an excess of acidic amino acids comparable to cells of H. halobium /38/. In contrast to other eubacteria these anaerobes have high cytoplasmic concentrations of inorganic ions /38/. As "normal proteins" would not be stable under these conditions, these bacteria have acidic proteins. Thus a general relation between the amino acid composition of proteins and the ion concentration in their surrounding microenvironment seems to be realized in archaebacteria as well as in certain eubacteria.

Cell envelope. Little information is available on the influence of high salt concentrations on structures of the cell envelope in halophilic eubacteria. In the Gram-negative, non-halophilic E. coli, however, detailed genetic studies have revealed, that the composition of the outer membrane is subject to osmotic regulation (see /16/). The biosynthesis of outer membrane proteins encoded by *ompC* and *ompF* in E. coli is strikingly affected by the osmolarity of the medium and *ompC* is preferentially expressed at high osmolarity while *ompF* is preferentially expressed at low osmolarity. A third locus, the *ompB* operon has been identified as a regulator region for both *ompC* and *ompF* that encodes two genes, *ompR* and *envZ*. A general role of *EnvZ* as an osmosensor is suggested by findings that mutations affecting transcription of *lamB* and *malE* map identical with *envZ* mutations, and that mutations in *envZ* also reduce the production of a periplasmic alkaline phosphatase and several iron-induced

outer membrane proteins /39-41/. A further gene, *micF*, is involved in the regulation of the *ompF* expression /42/. Results indicate the coregulation of *micF* with *ompC* in a way that *micF* acts to repress *ompF* under conditions favorable for *ompC* expression, i.e. at high osmolality /43/. Similar osmotically regulated structural changes have so far not been observed in halophilic eubacteria, although one has to predict that adaptative changes occur.

**Lipids.** It is evident that both moderately and extremely halophilic eubacteria have the capacity to regulate the composition of their membranes in regard to lipid head groups and fatty acids in response to the salinity of their surrounding environment. A general tendency in representatives of Gram-negative and Gram-positive eubacteria is an increase of negatively charged phospholipids with increasing salinity. In particular a decrease in the relative content of phosphatidylethanolamine was found in some bacteria, while the relative contents of phosphatidylglycerol and/or cardiolipin increased /44-47/.

**Osmotic adaptation.** A great variety of solutes is used within different eubacteria to achieve osmotic balance with the external environment. The ions outside the cell in most cases strongly inhibit enzymatic processes in the cytoplasm and accumulated substances generally differ from the major solutes outside the cells. In eubacteria, accumulation of high concentrations of inorganic ions, but not of organic solutes has been observed only in some anaerobes by Oren /38/. In most of the halophilic eubacteria, various organic solutes are accumulated. The kind of solute accumulated depends on growth conditions (in particular the osmolality of the growth medium) and, to the major part, on the bacterium. The major solute accumulated seems to be one important factor determining the degree of salt stress tolerated by the respective bacterium /16,48/. For some of these solutes, which were found as the major compatible solutes only in non-halophilic and slightly halophilic bacteria, their solubility and/or toxicity restrict accumulation in high concentrations. The solutes which predominate in halophilic, and halotolerant eubacteria are listed below:

- i) Non-halophilic and slightly halophilic eubacteria accumulate glutamate, proline, a few other amino acids, potassium, and several sugars (sucrose, fructose, glucose, trehalose).
- ii) Moderately and extremely halophilic and halotolerant eubacteria accumulate as major components glycine betaine or glutamate betaine, glucosylglycerol, in some cases also ectoine, and one or more minor components (glutamate, potassium, trehalose, sucrose).

This accumulation is either achieved by biosynthesis or by uptake from the medium. There is evidence that the presence of complex nutrients or other specific compounds, that can be taken up and accumulated by the bacteria, increase their salt tolerance (see /16/). This indicates, that accumulation of compatible solutes may be one limiting factor of the salt tolerance. Most of the information available on osmoregulatory responses and compatible solute accumulation is on non-halophilic and slightly halophilic eubacteria and far less on moderately and extremely halophilic eubacteria. (This topic has recently been reviewed /16/.)

In a number of non-halophilic and slightly halophilic eubacteria potassium is accumulated under conditions of osmotic stress. In *E. coli* two transport systems for potassium are regulated by the external osmolality or the turgor pressure, such as the Trk- and the Kdp-systems. The Kdp-system is in addition regulated on the genetic level, i.e. it is induced by decreasing the turgor pressure.

Glutamic acid is, under normal conditions, one of the major amino acids in the cellular amino acid pool of bacteria. The proportion of glutamic acid to the total cellular free amino acid pool is normally about 50%, but may increase, up to more than 90% under salt stress conditions. Its concentration is dependent on the osmolality of the growth conditions in a number of slightly and moderately halophilic bacteria and it can be accumulated by biosynthesis. As an anion its accumulation is dependent on the presence of equivalent concentrations of cations in the cytoplasm. It has also been shown that uptake of potassium and glutamic acid (or other weak acids) stimulate each other, and are in turn stimulated by the osmolality of the medium. It is reasonable to assume that potassium to a great part neutralizes the charges of glutamic acid.

Proline is a very soluble and suitable compatible solute that is, under normal conditions, present in the cytoplasm only in very minor amounts and whose biosynthesis is strictly feedback inhibited in many bacteria. Proline accumulation by biosynthesis has been shown in *Bacillus subtilis* var. *niger* and in *Streptomyces griseus*. Most of the other bacteria depend on active uptake systems for accumulation of proline. In *E. coli*, proline is taken up from the medium by several transport systems, two of which are activated by osmotic stress. If proline is present in the media these bacteria preferably accumulate this amino acid instead of glutamate and potassium.

Glycine betaine is the major compatible solute in many moderately and extremely halophilic eubacteria /48-51/. The first evidence for biosynthesis of glycine betaine in bacteria was obtained by Galinski and Trüper /49/. They studied the halophilic Ectothiorhodospira halochloris which accumulates this solute in concentrations exceeding 2 M. Betaine has been shown by gas chromatographic analysis and by  $^{13}\text{C}$ -NMR techniques to be one of the major solutes in a number of moderately halophilic eubacteria, that accumulate betaine in dependence on the salinity of growth conditions /48/. Even in E. coli, osmotically activated transport systems that catalyze the accumulation of glycine betaine have been described recently. The function of these transport systems is currently under debate. They do not seem to be strictly specific for betaine or proline, but their catalyzed reaction is apparently dependent on experimental conditions. Two independent transport systems have been described as osmotically induced proline transport systems, but later shown to prefer glycine betaine as the substrate.

These findings and the fact that a number of soluble proteins of these bacteria are severely inhibited by even low salt concentrations, makes it very likely that the cytoplasm of moderately and extremely halophilic eubacteria is not highly ionic. This is in contrast to the situation in the extremely halophilic archaeobacteria, that accumulate high internal potassium concentrations and have soluble proteins and a protein synthesizing machinery that is structurally adapted to the high ionic content of the cytoplasm. A similar adaptation of the cytoplasmic components to highly ionic conditions, however, has to be expected in those halophilic anaerobes that lack organic compatible solutes and that have a highly ionic cytoplasm, such as Haloanaerobium, Halobacteroides, and Clostridium lortetii /38/.

#### CONCLUSION

As we have seen, different approaches have been developed for adaptation to high salt concentrations. In halobacteria and also in a few eubacteria the cytoplasm is highly ionic and potassium is the predominant cytoplasmic cation. In most of the eubacteria, and in the alga Dunaliella, however, the external ions are largely excluded and organic solutes are accumulated either by uptake or by biosynthesis. In both cases the function of physiological processes within the cytoplasm seems to be adapted to high concentrations of the solute accumulated. Apparently one general strategy is the increase of negative charges at the surface of molecules (lipids and proteins) exposed to highly ionic solutions.

There are, however, a number of unanswered crucial questions. We know nothing about the signals that trigger adaptation processes following salt stress, nor do we know how this signal is transformed into the adaptive processes. The structural and/or functional properties that determine the observed salt optima in various halophilic bacteria are also unknown. It is unlikely that accumulation of sufficiently high concentrations of suitable compatible solutes determines the halophilic property of a bacterium. There are several known cases where accumulation of compatible solutes proceeds towards salt concentrations higher than optimal and where solute accumulation is one of the factors determining the upper limit of growth rather than the optimum (see /16/). A crucial point in this respect may be found in structural and functional adaptation of membrane and envelope components. Our present knowledge, however, does not allow any clear conclusions.

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